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Production of 5-keto-D-gluconate by acetic acid bacteria is catalyzed by pyrroloquinoline quinone (PQQ)-dependent membrane-bound D-gluconate dehydrogenase [1]

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Abstract

Gluconobacter suboxydans IFO 12528 was selected as the best strain for 5-keto-D-gluconate (5KGA) production by oxidative fermentation. 5KGA was markedly accumulated by the strain during cultivation in a medium containing D-glucose and/or D-gluconate. The resting cells and the membrane fraction also catalyzed 5KGA formation with a minimal formation of 2-keto-D-gluconate (2KGA), an alternative keto-D-gluconate from D-gluconate. The membrane fraction of the organism was confirmed to contain a membrane-bound D-gluconate dehydrogenase (GADH) catalyzing D-gluconate oxidation to 5KGA of which optimum pH and temperature were found at pH 4 and 15°C, respectively. After treating the membrane fraction with EDTA allowing conversion from holo-GADH to the apoenzyme, 5KGA-forming GADH was confirmed to be a pyrroloquinoline quinone (PQQ)-dependent enzyme by the fact that the enzyme activity was restored by the addition of $CaCl_2$ and PQQ. The 5KGA-forming GADH was totally distinct from 2KGA-forming GADH in which a covalently bound FAD functions as coenzyme. 5KGA-forming GADH was well solubilized from the membrane fraction with *n*-octyl-*β*-D-thioglucoside and 5KGA formation was favourably catalyzed at relatively lower temperature, while 2KGA-forming enzyme was solubilized with Triton X-100 and relatively higher temperatures was optimum for 2KGA formation. These results are completely discrepant from the conclusion proposed by Klasen et al. [R. Klasen, S. Bringer-Mayer, H. Sahm, J. Bacteriol., 177, 1995, 2637] claiming that 5KGA was produced by D-gluconate oxidation catalyzed by NADP-dependent cytoplasmic 5KGA reductase from *Gluconobacter* species at fairly alkaline pH such as 10.

Keywords: Acetic acid bacteria; *Gluconobacter suboxydans*; 5-Keto-D-gluconate; Oxidative fermentation; PQQ-dependent dehydrogenase

1. Introduction

Oxidation of various alcohols, sugars, and sugar alcohols catalyzed by aerobic Gram negative microorganisms such as acetic acid bacteria or pseudomonads is known as the oxidative fermentation. Of the two genera of acetic acid bacteria, *Gluconobacter* species catalyze oxidation of sugars and sugar alcohols such as D-glucose, D-fructose, D-gluconate, D-sorbitol, and glycerol. By contrast, *Acetobacter* species have a high activity of ethanol oxidation [1]. Many

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[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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different oxidizing systems of which enzyme activity is coupling with the respiratory chain of acetic acid bacteria have been demonstrated [2].

5-Keto-D-gluconate (5KGA) is a compound formed by only acetic acid bacteria when grown on a medium containing D-glucose and/or D-gluconate. *Gluconobacter* strains produce two keto-D-gluconates, 5KGA and 2-keto-D-gluconate (2KGA), from D-glucose via D-gluconate. Some attempts to produce 5KGA have been made with *Gluconobacter* strains as this compound was reported to be useful for production of L-ascorbic acid by Gray's method [3–7]. Moreover, it was reported that 5KGA might be a precursor for tartrate from D-glucose with *Gluconobacter suboxydans* [8]. But there is little biochemical information about 5KGA formation regarding to the properties of the enzyme concerned. Thus, the localization, catalytic properties, and nature of the prosthetic group of the enzyme has remained to be clarified. Among 5KGA metabolizing enzymes in *Gluconobacter* strains, 5KGA reductase (5KGR) is found designating its physiological role to be in reduction of 5KGA to D-gluconate in cytoplasm putting it into the pentose-phosphate pathway after phosphorylation of D-gluconate to 6-phospho-D-gluconate [9].

D-Gluconate dehydrogenase (GADH) catalyzing 2KGA formation has been purified from the membrane fraction of *Gluconobacter dioxyacetonicus* IFO 3271 and well characterized to be an enzyme complex of a covalently bound flavin and cytochrome *c* [10]. It shows almost the same properties as those of purified enzymes from *Pseudomonas aeruginosa* [11,12], including subunit structure, heme content and optimum pH of the enzyme reaction. 2KGA dehydrogenase (2KGDH), which catalyzes formation of 2,5-diketo-D-gluconate from 2KGA, has been purified and characterized from *Gluconobacter melanogenus* [13]. GADH and 2KGDH have almost the same enzyme substructure consisting of three different subunits, namely, dehydrogenase, cytochrome *c*, and a third subunit whose function is unknown.

The cytoplasmic NADP-dependent 5KGR has been purified from *G. suboxydans* [9], but the optimum pH for D-gluconate oxidation is around 10 by the reduction of NADP. Klasen et al. [14] recently purified 5KGR from *G. oxydans* and sequenced the 5KGR gene concluding that 5KGR is the responsible enzyme for 5KGA production. Such high alkaline pH is not favourable for 5KGA production in the oxidative fermentation by acetic acid bacteria most of which are done under fairly acidic conditions [2]. Furthermore, if the 5KGA production is carried out in the cytoplasm, the organism has to incorporate the substrate, D-gluconate, into the cells and then pumps out the product, 5KGA, under expense of lots of bioenergy. Such energy wasting mechanism looks unfavourable for the proposed mechanism of oxidative fermentation.

D-Sorbitol dehydrogenase from *G. suboxydans* subsp. var. α IFO 3254 was purified and characterized to be a heterotrimeric flavohemoprotein containing covalently bound FAD as the primary coenzyme in the subunit I and cytochrome *c* in the subunit II [15]. Recently, occurrence of another D-sorbitol dehydrogenase of heterotrimer has been indicated with *G. suboxydans* KCTC 2111 (= ATCC 621) in which pyrroloquinoline quinone (PQQ) functions as the primary coenzyme in the subunit I [16]. Judging from the occurrence of two different types of D-sorbitol dehydrogenase in *G. suboxydans*, it is reasonable to suggest the presence of another GADH which contributes to 5KGA production in the cytoplasmic membrane of acetic acid bacteria.

In order to make clear that 5KGA is produced by a membrane-bound alternative GADH distinct from 2KGA-forming enzyme, 5KGA formation was demonstrated under different manners using the growing cells, the resting cells, the membrane fraction, and the detergent solubilized enzymes. The optimum pH for 5KGA formation was reconfirmed by this study to be at acidic conditions as reported previously [17]. It showed a clear contrast to the conclusion by Klasen et al. [14]. The second finding with

5KGA-forming GADH was indicated by the fact that the enzyme is PQQ-dependent, unlike 2KGA-forming GADH. Resolution of the holo-GADH to apo-GADH and subsequent reconstitution of the holoenzyme with exogenous addition of PQQ and $CaCl_2$ restoring 5KGA formation were also demonstrated. Distinct from 5KGR, a series of clear evidence that 5KGA-forming GADH catalyzes one directional oxidation of D-gluconate to 5KGA irreversibly and GADH activity may link to the respiratory chain in the cytoplasmic membrane are presented.

2. Experimental

2.1. Chemicals

Sodium D-gluconate and sodium 2KGA were supplied by courtesy of Dr. H. Imanaka, Fujisawa Pharmaceutical, Osaka. Potassium 5KGA was a product from Sigma. NAD, NADP, their reduced forms, and yeast extract were kindly donated from Oriental Yeast, Tokyo. PQQ was prepared to be absolutely pure form as described previously [18]. *n*-Octyl-β-D-thioglucoside (OT) was a product from Dojindo Laboratories, Kumamoto.

2.2. Microorganism and culture conditions

G. suboxydans IFO 12528 was donated by the Institute for Fermentation, Osaka (IFO), and used throughout this work. For preparation of membrane fraction, culture medium consisted of 20 g of sodium D-gluconate, 5 g of D-glucose, 3 g of glycerol, 3 g of yeast extract, and 2 g of polypeptone in 1 l of tap water. The pH of the medium was spontaneously settled to 6.5 when all the ingredients were mixed. A flask culture was made on 100 ml of the medium in a 500-ml Erlenmeyer flask under continuous shaking at 30°C. A large scale culture was done on 30 l of the medium in a 50-l jar fermenter with aeration under vigorous agitation at 30°C. To investigate keto-D-gluconate accumulation in the culture medium during the growth, a culture medium having the following ingredients was used: 10 g of sodium D-gluconate, 10 g of D-glucose, 2 g of polypeptone and 1 g of yeast extract in 1 l of tap water. The cultivation was made on 20 ml of the medium in a 100-ml Erlenmeyer flask.

2.3. Cell fractionation

The harvested cells were suspended in 10 mM potassium phosphate buffer (KPB), pH 6.0, and centrifuged at $20{,}000 \times g$ for 10 min at 4°C. The precipitated cells were resuspended with the same buffer at the ratio of 1 g of wet cells per 5 ml of the buffer. After successive two times of passing through a French pressure cell press at 1,000 kg/cm^2, the cell homogenate was centrifuged at $8{,}000 \times g$ for 5 min to remove the intact cells. The resulting supernatant was further centrifuged at $68{,}000 \times g$ for 60 min and the membrane fraction was collected as the precipitate. When complete removal of cytoplasmic soluble proteins from the membrane fraction was necessary, the membrane fraction was resuspended in 10 mM KPB, pH 6.0, homogenized in a glass homogenizer, and repeated the same operation of ultracentrifugation as above. The protein content was determined by a modification of Lowry's method [19]. A commercial bovine serum albumin (fraction V) was used as the standard.

2.4. Quantitative determination of 5KGA and 2KGA

5KGA and 2KGA accumulated in the culture medium or formed in the reaction mixtures by the membrane fraction were determined enzymatically using 5KGR and 2KGA reductase (2KGR). Since 5KGR is only specific for 5KGA and 2KGR is for 2KGA, a specific determination of individual keto-D-gluconates was done under essentially the same method described previously [20,21].

The reaction mixture (1 ml) for keto-D-gluconate formation contained 200 μmol of sodium

D-gluconate, 0.5 ml of the buffer and the membrane fraction, and was incubated with shaking at 25°C for 2 h. The membrane fraction was suspended in 2 mM acetate buffer, pH 5.0. McIlvaine buffer, pH 3.0, was basically used for 5KGA production. The reaction mixture was centrifuged at $180{,}000 \times g$ for 30 min to remove the membrane after incubation. The amount of 2KGA and 5KGA in the supernatant was determined enzymatically as described above, and expressed as micromole of keto-D-gluconate formed per milligram of protein.

2.5. Enzyme assay

GADH activity was measured by the method of Wood et al. using ferricyanide as an electron acceptor as described previously [13,15]. One unit of the enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of substrate per minute under the conditions. Enzyme activity was determined with a solubilized fraction with or without reactivation by PQQ and calcium ions.

D-Gluconate oxidizing activity with the membrane fraction was measured polarographically with an oxygraph equipped with a Clark type oxygen electrode (Yellow Springs, OH, USA) at 25°C. The reaction mixture (2.5 ml) contained 1.0 ml of the buffer, 100 μmol of sodium D-gluconate and the membrane fraction. The oxidation rate was expressed as 1 μmol of oxygen consumed per minute under the conditions.

2.6. Resolution of holo-GADH and reactivation of apo-enzyme by PQQ and metal ions

The membrane fraction was suspended in 10 mM phosphate buffer, pH 7.0, containing 10 mM EDTA. After stirring for 30 min in an ice bath, the membrane suspension was centrifuged at $100{,}000 \times g$ for 30 min and the precipitated membrane was resuspended and homogenized in 10 volumes of the same buffer, and then centrifuged again as above. The resulting precipitate designated as EDTA-treated membrane involving apo-GADH was homogenized by a glass homogenizer in an adequate volume of 2 mM acetate buffer, pH 5.0.

The EDTA-treated membrane suspension was added to 10 mM acetate buffer, pH 5.0, 15 μM PQQ, and 10 mM metal ions, of which counter ion of chloride were used. The mixture was incubated for 15 min at 25°C and then a portion of the mixture was used for keto-D-gluconate production as the holo-GADH containing membrane fraction.

3. Results and discussion

3.1. Fermentative production of 5KGA

Since *G. suboxydans* IFO 12528 was indicated to be useful for 5KGA formation in the previous study [17], the same strain was used throughout this study. Cultivation was made in the medium containing D-glucose and D-gluconate as carbon sources at 30°C with rotary shaking or static condition. The total cell yields from shaking culture was better than those from static culture. Although the medium gave less cell yields than the other medium containing glycerol, the medium was found to be the most suitable for 5KGA formation as described in the previous paper [17]. As shown in Fig. 1, pH of the culture broth came down to below pH 4 and 5KGA was accumulated with an yield of more than 80% to the initial carbon source within 24 h under shaking. Under static culture, the rate of 5KGA accumulation was rather slow compared to the shaking culture because of the less yield of cell growth. However, the final conversion ratio from D-gluconate to 5KGA was over 90%. Contrary, 2KGA accumulation was slight in both cases. Although a longer cultivation time was required to get the maximum 5KGA production under static culture, the productivity of 5KGA was higher than that of shaking culture.

We classified the strains belonging to the genus *Gluconobacter* into three groups basing

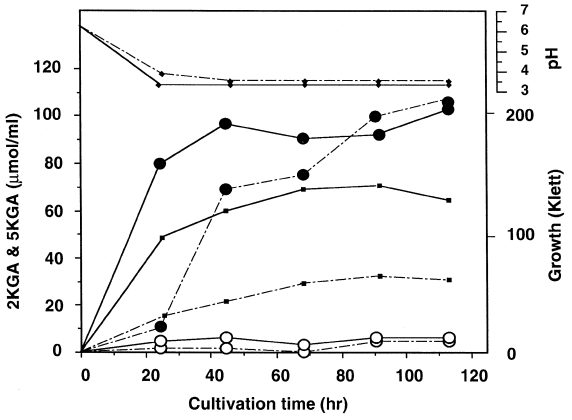

Fig. 1. Time course of keto-D-gluconates production by *G. suboxydans*. The organism was cultured in the medium containing 1% D-glucose and 1% D-gluconate as carbon sources. The cultivation was made on 20 ml of the medium in a 100-ml Erlenmeyer flask with rotary shaking (—) or static conditions (— – —) at 30°C. The amounts of 5KGA and 2KGA in the culture broth were determined enzymatically as described in Section 2. Symbols: ●, 5KGA; ○, 2KGA; ■, growth; ♦, pH of culture broth.

on 5KGA/2KGA ratio accumulated in the medium: namely, 5KGA producer, 2KGA producer, and the intermediate type producing both 2KGA and 5KGA in nearly the same amount. It is worth to make sure whether such variations come from a specific regulation of GADH activity or two distinct enzymes function in the organism, though the extent of enzyme activity largely depends on culture conditions. The previous data indicating that *G. suboxydans* IFO 12528 is 5KGA producer [17] were reconfirmed in this experiment.

3.2. Optimum conditions for 5KGA formation with membrane fraction

The growth of *G. suboxydans* varied depending on the carbon source used for the growth medium. A clear increase in cell yield was observed by the addition of glycerol to a medium containing D-glucose and D-gluconate as the main carbon source. Therefore, the addition of glycerol was useful to get the increased cell masses. However, a lowered productivity of 5KGA was always observed by the resting cells from glycerol containing medium [17]. In this work, the membrane fraction from *G. suboxydans* was prepared from the cells harvested at the early stationary phase in the medium containing D-glucose, D-gluconate, and glycerol. This membrane fraction produced more amount of 2KGA than the membrane prepared from the medium excluding glycerol. Considering these results together with the previous study [17], 5KGA formation became predominated when a membrane fraction prepared from the cells harvested at the late stationary phase grown in the medium without glycerol. Thus this membrane fraction was useful to clarify the difference between the two types of keto-D-gluconate forming GADHs.

5KGA formation by the membrane fraction was carried out under various pHs. The optimum pH for 5KGA formation was found to be at acidic range, pH 2.5–4.0. 2KGA was also produced in a broad acidic range around pH 5 and the amount of 2KGA produced at pH 5 was about a third of that of 5KGA at pH 3. The optimum production of 5KGA at acidic pH region was similar to the results with the resting cells. A considerable 5KGA production was also observed at pH 9. However, it is unclear whether the enzyme producing 5KGA at acidic pH could exert the same function at pH 9. There is a clear observation that a buffer solution involving glycine as an ingredient was found to be favourable for 5KGA formation at around pH 9, while no 5KGA formation was observed when Tris-buffer was used instead. It was also clear that neither a reasonable amount of NADP nor 5KGR activity were detected with the membrane fraction used in the experiment. To give a correct answer to this question why the membrane fraction catalyzed 5KGA formation at pH 9, it must be checked with purified enzyme. Solubilization and further purification of 5KGA-forming GADH are now under progress.

After treating the membrane fraction with PQQ and $CaCl_2$ to confirm holoenzyme formation, the production of keto-D-gluconates at various pHs was investigated. As shown in Fig. 2, the productivity of 5KGA increased about two

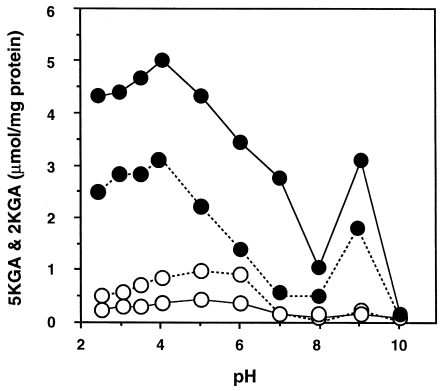

Fig. 2. Effect of pH on 5KGA production with membrane fraction from *G. suboxydans*. The membrane fraction was prepared from the cells harvested at the early stationary phase in the medium containing D-glucose, D-gluconate, and glycerol as carbon sources. The membrane fraction was prepared as described in Section 2. Before the reaction for keto-D-gluconates production the membrane fraction was incubated with (—) or without (· · ·) 15 μM PQQ and 10 mM $CaCl_2$ for 15 min at 25°C. The reaction mixture (1 ml) containing 200 μmol of sodium D-gluconate, the membrane fraction, and 0.5 ml of the buffer of various pHs was incubated for 2 h at 25°C with shaking. McIlvaine buffer was used for pH 2.5–8.0 and 0.1 M glycine–NaOH buffer was used for pH 9.0–10.0. After incubation the membrane was removed by centrifugation at 180,000 × *g* for 30 min and the amounts of 5KGA (●) and 2KGA (○) in the supernatant were determined. The value are expressed as the amount of keto-D-gluconates per milligram protein of membrane fraction.

times by exogenous PQQ at pH 3. Contrarily, 2KGA production went down by the presence of PQQ and $CaCl_2$ less than the original levels. The conversion ratio of D-gluconate to 5KGA and to 2KGA at pH 3 raised over 10 times higher with the membrane fraction to which PQQ had been added. Thus, it was concluded that 5KGA production was catalyzed by the enzyme having PQQ as the coenzyme and that 5KGA-forming enzyme was present partially as apoenzyme during cultivation and subsequent cell fractionation. When the specific productivity calculated as the amount of 5KGA formed per mg protein at pH 3, it was about 4.5. This value was comparable to that by the resting cells harvested from the mid-exponential phase [17], suggesting that 5KGA could be produced by the enzyme located in the membrane fraction but not by the cytoplasmic enzyme. Exogenous addition of NADP to the washed membrane fraction gave no appreciable 5KGA production.

Effect of temperature on 5KGA production was investigated over a temperature range of 5°C to 40°C incubating the reaction mixture without shaking. The lower productivity of keto-D-gluconates in this experiment probably caused by the static incubation. The production of 5KGA increased as the incubation temperature decreased and 5KGA was maximally produced at 15°C as shown in Fig. 3. In contrast to 5KGA production, 2KGA production increased as the incubation temperature was elevated showing the highest 2KGA yield at 35–40°C. The incubation at 15°C gave the maximum amount of 5KGA and scarce amount of 2KGA, that was reciprocal to the case of 2KGA production. 2KGA production exceeded 5KGA as the incubation was made at relatively higher temperatures over 40°C.

For the maximum 5KGA production with the membrane fraction, incubation must be carried out at lower temperature such as 15°C under acidic pH around 3. Under these conditions, formation of 2KGA could be controlled to be minimum. These results were highly consistent with the data done by the resting cells. At the same time, it has been confirmed that 5KGA

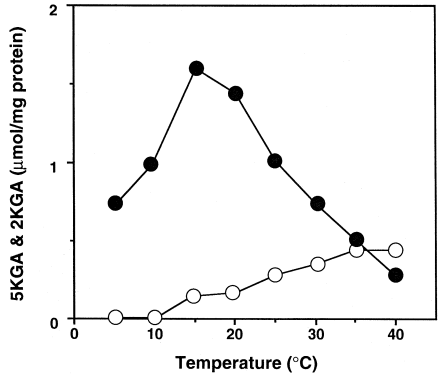

Fig. 3. Effect of temperature on 5KGA production with membrane fraction from *G. suboxydans*. The reaction mixture (1 ml) containing 200 μmol of sodium D-gluconate, the membrane fraction, and 0.5 ml of McIlvaine buffer, pH 3.0, was incubated at various temperatures without shaking. After incubation for 2 h, the yields of 5KGA (●) and 2KGA (○) in the supernatant were determined and the values are expressed as described in Fig. 2.

formation is catalyzed by the enzyme located in the cytoplasmic membrane.

3.3. D-Gluconate oxidizing enzyme and keto-D-gluconates formation

It was indicated that the membrane fraction from *G. suboxydans* produced 5KGA and 2KGA from D-gluconate. D-Gluconate oxidizing activity was investigated with the membrane fraction under different pHs. The enzyme activity was calculated by a dual method, initial velocity measurement of oxygen uptake and analysis of oxidation products as mentioned in Section 2. In general, acidic pHs were found to be favourable for the formation of keto-D-gluconates. Comparing the pH profiles vs. D-gluconate oxidizing activity to keto-D-gluconate production with the membrane fraction, the optimum pH from the both assays was the same. However, the results obtained at higher pHs were different: i.e., although D-gluconate oxidizing activity was not detected at higher pH, keto-D-gluconates production was detected when the reaction mixture was served as the substrate for 2KGR and 5KGR. Oxidizing activity was measured from the initial velocity measurement of oxygen uptake within a few minutes after the reaction started, while the amount of keto-D-gluconates produced was analyzed after 2 h incubation. Therefore, keto-D-gluconates could be detected after the long period of incubation, even if the incubation was done at unfavourable higher pHs.

Exogenous addition of both PQQ and $CaCl_2$ caused further increase in oxidizing activity as typically shown at pH 4 by three times elevation of D-gluconate oxidizing activity (Fig. 4). The increase in D-gluconate oxidizing activity allowing higher 5KGA production by holoenzyme formation with PQQ and $CaCl_2$ indicated that, in this experiment, the enzyme activity exerted contribution mainly to the formation of 5KGA but not 2KGA formation. These results also strongly gave an evidence that the primary dehydrogenase in D-gluconate oxidation to 5KGA was PQQ-dependent.

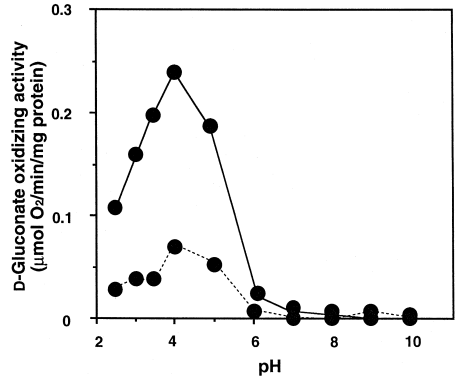

Fig. 4. Effect of pH on D-gluconate oxidizing activity of membrane fraction from *G. suboxydans*. Oxidizing activity for D-gluconate was measured with a Clark-type oxygen electrode. The reaction mixture (2.5 ml) containing 100 μmol of sodium D-gluconate, the membrane fraction, and 1.0 ml of the buffer. The buffer used was the same as described in Fig. 2. D-Gluconate oxidizing activity was determined with the membrane fraction before (· · ·) and after (—) incubation with 15 μM PQQ and 10 mM $CaCl_2$ for 15 min at 25°C.

Many different dehydrogenases oxidizing alcohols and sugars such as alcohol, aldehyde, D-glucose, D-fructose, glycerol, D-sorbitol, and 2KGA, have a function as the primary dehydrogenase in the sugar oxidase respiratory chain in acetic acid bacteria and other oxidative bacteria generating bioenergy during substrate oxidation [2]. These dehydrogenases have been purified from the membrane and well characterized. 2KGA-forming GADH has been already purified from *G. dioxyacetonicus* IFO 3271, a typical 2KGA producer [10]. The 2KGA-forming GADH contains a covalently bound flavin as the coenzyme contrary to the 5KGA-forming GADH which was confirmed to be a PQQ-dependent enzyme in this study.

3.4. Resolution and restoration of 5KGA formation and D-gluconate oxidizing activity

The treatment of the membrane suspension with EDTA caused the decrease of 5KGA productivity. It was restored by the addition of PQQ and $CaCl_2$. The addition of PQQ to the EDTA-treated membrane did not restore 5KGA production, whereas the addition of $CaCl_2$ re-

stored 5KGA production to the original level of the native membrane but not to the maximum level observed by the addition of PQQ and $CaCl_2$. This means that EDTA removed calcium ions and PQQ still existed in the membrane after EDTA treatment. The addition of both PQQ and $CaCl_2$ showed nearly the same level of 5KGA formation with both EDTA- and non-treated membranes. On the other hand, 2KGA production increased with EDTA-treated membrane and decreased by the addition of $CaCl_2$ as shown in Fig. 5A. These results showed that calcium ions gave a negative effect on 2KGA production reciprocal to 5KGA production. The differences in 2KGA and 5KGA formations was made clear from the results obtained with EDTA-treated membrane, that is, 5KGA-forming GADH is distinct from 2KGA-forming GADH.

D-Gluconate oxidizing activity was also affected by treating the membrane fraction with EDTA. D-Gluconate oxidizing activity decreased more remarkably than that of 5KGA production (Fig. 5B). Addition of either $CaCl_2$ or PQQ separately to the EDTA-treated membrane was not so effective as seen in 5KGA production. The addition of PQQ and $CaCl_2$ to the EDTA-treated membrane gave about the same activity as that observed with non-treated membrane in the presence of PQQ and $CaCl_2$. The EDTA treatment was considered to convert GADH to the apoenzyme and only a little D-gluconate oxidizing activity was seen with such the membranes alone. The conditions for the restoration of D-gluconate oxidizing activity was very similar to that of 5KGA production but not 2KGA production, namely, no restoration by only PQQ, a weak restoration by $CaCl_2$, and full restoration by PQQ and $CaCl_2$. By these results, it was evident that D-gluconate oxidizing activity responsible for 5KGA production was only sensitive to EDTA-treatment.

The effect of metal ions on restoration of 5KGA formation was investigated with various divalent cations that used commonly for holoenzyme formation of quinoproteins in the presence of PQQ [22–24]. The highest restoration was observed with calcium ions in the presence of PQQ as shown in Fig. 6. Nickel, cobalt and magnesium ions also showed some reactivation and they showed the almost same extent. Other divalent cations showed no effect on 5KGA production. It was interesting to see that the metal ions showing a stimulative effect on

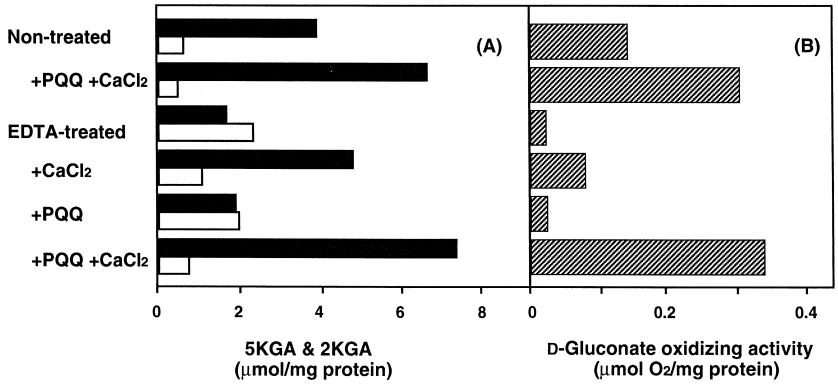

Fig. 5. Restoration of 5KGA formation (A) and D-gluconate oxidizing activity (B) of EDTA-treated membrane fraction by PQQ and $CaCl_2$. The EDTA-treated and non-treated membrane were incubated with or without 15 μM PQQ and/or 10 mM $CaCl_2$ for 15 min at 25°C, and a portion of the mixture was used for keto-D-gluconate production and the measurement of D-gluconate oxidizing activity. (A) The formation of keto-D-gluconate was performed in the reaction mixture (1 ml) containing 200 μmol of sodium D-gluconate, 0.5 ml of McIlvaine buffer, pH 3.0, and the membrane fraction at 25°C for 2 h. The amounts of 5KGA (filled rectangle) and 2KGA (rectangle) were determined as described in Section 2. (B) D-Gluconate oxidizing activity was measured at pH 3.0 with an oxygen electrode at 25°C under the conditions described in Fig. 4.

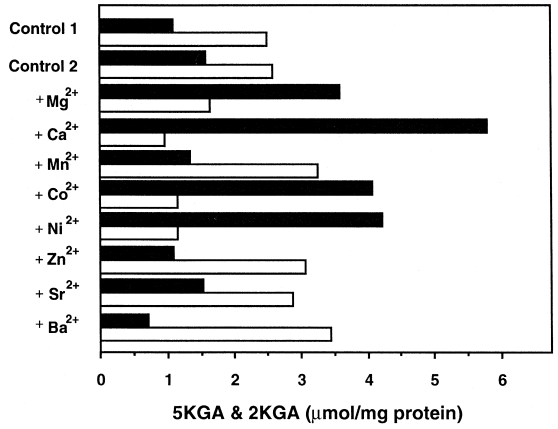

Fig. 6. Effect of metal ions on restoration of 5KGA formation of EDTA-treated membrane fraction in the presence of PQQ. The EDTA-treated membrane was incubated as described in Fig. 5 using 10 mM various metal ions, of which counter ions of chloride were used. The formation and determination of 5KGA (filled rectangle) and 2KGA (rectangle) were performed as described in Fig. 5. Control 1 means incubation of the membrane fraction without PQQ and metal ion. Control 2 means that performed with PQQ and no metal ions.

5KGA production resulted some inhibitory effect or no effect on 2KGA production. The species of effective metal ions for the restoration of 5KGA production coincided with those of other quinoproteins such as D-glucose dehydrogenase [22] and alcohol dehydrogenase [25].

3.5. Solubilization of keto-D-gluconate forming enzymes

Differential solubilization of the two keto-D-gluconate forming GADHs was attempted by the aid of detergents. When the membrane fraction (10 mg protein/ml) was treated with 1% OT for 1 h, solubilization of 5KGA-forming GADH was predominated. When relationship of GADH activity measured at pH 3.0 to incubation temperatures was examined, 5KGA formation increased as the incubation temperatures were controlled at rather lower levels such as below 20°C as shown in Fig. 7. The optimum temperature was found at 15°C as similarly shown in Fig. 4. Stimulation of 5KGA-forming enzyme activity by the addition of $CaCl_2$, PQQ, and $CaCl_2$ and PQQ, as similarly observed in the above experiments. When the enzyme solubilization was done with 2% Triton X-100 for 3 h, GADH activity exerted at rather high temperatures more than 30°C and the composition of the products was much changed. As expectedly, 2KGA was increased in the reaction mixture as similarly seen in Fig. 3. These results also clearly indicated the presence of two different types of GADHs in the membrane fraction of *G. suboxydans*. As briefly mentioned above, the contents of the two GADHs must be regulated depending on culture conditions of which mechanism is unclear at the moment.

Enzyme solubilization was attempted with the membrane fraction which had been treated with EDTA as described above. The resulting GADH solubilized with OT gave a slight enzyme activity when measured under the standard assay conditions, indicating that the most of GADH was converted to apoenzyme. With such less active enzyme, restoration of the enzyme activity was checked by adding PQQ, $CaCl_2$, and PQQ and $CaCl_2$. Different from the results obtained with the native non-treated membrane, influence by the exogenous addition of both PQQ and $CaCl_2$ became more evident (data now shown).

Further purification of 5KGA-forming GADH is under processing to characterize the enzyme

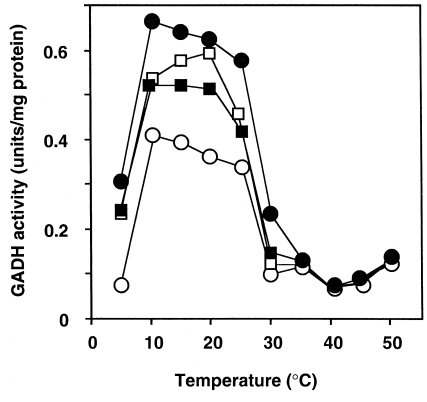

Fig. 7. Effect of temperature on GADH activity solubilized from the membrane fraction. GADH was solubilized with 1% OT from the membrane fraction. Enzyme activity was assayed with potassium ferricyanide as an electron acceptor at pH 3.0 with the solubilized enzyme before (○) and after incubation with PQQ (□), $CaCl_2$ (■), and PQQ and $CaCl_2$ (●).

in detail and to reconstitute the D-gluconate oxidizing activity from the essential components separately isolated from the organism. If a regulatory mechanism giving only 5KGA by *G. suboxydans* could be successfully set by elaborating the culture conditions or by molecular genetic criteria, a mass production of 5KGA must become promising for the industrial applications requiring a constant supply of 5KGA. For such success, the results presented here must have an important meaning for further development in oxidative fermentation by acetic acid bacteria.

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